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14. ABSTRACT A polypurine tract (PPT) containing multiple GGA repeats in the HER-2/neu promoter is important to control HER-2/neu transcription. We investigated the ability of the PPT to form a G-quadruplex-related secondary structure using biochemical techniques and screened a small family of potential G-quadruplex ligands that could stabilize PPT secondary structure formation in solution. We evaluated several potential lead compounds for their ability to suppress HER-2/neu expression in breast cancer cells. Circular dichroism studies with the distal half of the PPT were similar to findings previously reported for tetrad:heptad DNA. DNA polymerase arrest assays demonstrated potassium induced arrest, and several G-quadruplex interactive compounds that stabilize the HER-2/neu PPT secondary structure were identified from this assay. Telomestatin and a lead compound in the fluoroquinolone class stabilize the HER-2/neu PPT secondary structure in solution and reduce HER-2/neu expression in breast cancer cells. We conclude the HER-2/neu promoter can form a stable secondary structure known as a tetrad:heptad in solution. Compounds that stabilize the tetrad:heptad were identified and some of them reduced HER-2/neu expression in breast cancer cells. Further studies are needed to fully characterize the secondary structure and link the effects of compounds on HER-2/neu expression to their direct interaction with the HER-2/neu promoter.					
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Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	11
Reportable Outcomes.....	11
Conclusions.....	11
References.....	11

Introduction

The central hypothesis of this project is that the formation of a tetrad:heptad variant of a G-quadruplex by the HER-2/neu promoter represses HER-2/neu transcription. The tetrad:heptad provides a target for selective drug-DNA interaction by G-quadruplex interactive compounds and inducing or stabilizing the tetrad:heptad in the HER-2/neu promoter with a G-quadruplex interactive compound will silence HER-2/neu expression.

The specific aims of this proposal are:

Specific Aim 1: Investigation of the potential biological role of a tetrad:heptad DNA structure in the HER-2/neu promoter in the regulation of HER-2/neu gene expression.

Specific Aim 2: Investigation of the ability of quadruplex selective DNA interactive compounds to target the tetrad:heptad structure in the HER-2/neu promoter *in vitro* and *in vivo*.

In order to accomplish these aims, we proposed a series of experiments to evaluate the ability of G-quadruplex interactive compounds to bind to the HER-2/neu promoter in solution and prevent HER-2/neu expression in cells. In our statement of work, we proposed to initiate these experiments in year 1 to identify lead compounds, and continue their characterization in years 2 and 3. In our statement of work, we proposed to initiate biochemical studies in year 2 to further characterize the structure formed by the HER-2/neu promoter in an attempt to understand the potential biological role of DNA secondary structure in the regulation of HER-2/neu expression. In this annual report, we describe our preliminary findings on the G-quadruplex interactive compounds that we screened for interaction with the HER-2/neu promoter and on further characterization of the DNA secondary structure formed by the polypurine tract in the HER-2/neu promoter.

Body

The HER-2/neu oncogene is frequently overexpressed in breast cancer and represents an important therapeutic target. HER-2/neu expression is frequently disproportionate to gene copy number with or without gene amplification as a result of transcriptional activation. A polypurine tract (PPT) containing multiple GGA repeats is an important promoter element in the control of HER-2/neu transcription. GGA repeats have been shown to form unusual DNA structures related to guanine (G) quadruplexes at physiological potassium concentrations. G quadruplexes are emerging as potential therapeutic targets for the treatment of cancer.

We propose that the PPT might be able to fold into an intramolecular quadruplex within cells. From previous studies it was known that insertion of GGA/TCC repeats into plasmid DNA makes that region sensitive to S1 nuclease, suggesting the formation of secondary DNA structure [1]. Recently NMR data showed that sequences containing four consecutive GGA repeats form a very stable quadruplex variant under physiological potassium concentrations [2, 3]. This structure, called a tetrad:heptad (**Figure 1**), is composed of a typical guanine tetrad stacked on a guanine-adanine heptad containing 4 guanines and 3 adenines. The purine rich tract of the HER-2/neu promoter contains the first 11 nucleotides of (GGA)₄ needed to form a tetrad:heptad and quadruplex formation could prevent the binding of essential transcriptional factors, such as Ets, thus decreasing

HER-2/neu transcription. Quadruplex formation in the HER-2/neu promoter might also be enhanced by the addition of DNA interactive compounds that selectively bind and stabilize quadruplex formation [4].

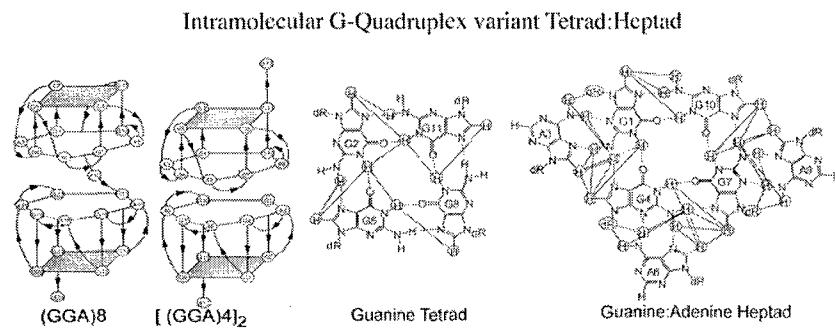


Figure 1. Tetrad:heptad DNA structures formed by oligos with four or eight GGA repeats.

To investigate if G-quadruplex interactive compounds are able to induce the secondary structure in the HER2/neu PPT, we used a modification of a published technique, the DNA polymerase (Taq Pol) arrest assay [5, 6]. The Taq Pol assay is based on the principle that intramolecular structures, such as G-quadruplexes, cause an arrest of DNA synthesis. When end-labeled primer is annealed to the template and dNTPs are added, Taq Pol initiates DNA synthesis, and the primer is extended to the end of the template or arrested at the site of a stable intramolecular secondary structure in the template strand (Figure 2A). The assay is carried out at physiological pH and has the advantage of using a low template concentration that favors intramolecular secondary DNA structure formation. Several G-quadruplex interactive compounds were identified from this assay that could stabilize the secondary DNA structure in the HER-2/neu PPT (Figure 2B and 2C). We evaluated two commercially available compounds, the cationic porphyrins TmPyP2 and TmPyP4 because the latter compound was shown to be a good potential G-quadruplex ligand, while the former compound is its positional isomer and binds less well to G-quadruplex DNA [7-10]. We also evaluated telomestatin, a natural product isolated from *Streptomyces anulatus* that is named for its ability to inhibit telomerase by stabilizing telomeric G-quadruplex and provided to one of us (LH) by Dr. Shin-ya (Tokyo University) [6, 11]. We also screened twelve proprietary compounds based on a common fluoroquinolone pharmacophore, because some fluoroquinolones had been shown to interact with G quadruplex DNA [12, 13]. These compounds were provided to us by Cylene Pharmaceuticals, Inc (San Diego, CA) through a materials transfer agreement, and had shown promise in G-quadruplex binding assays performed at Cylene (personal communication, Adam Siddiqui-Jain, Cylene Pharmaceuticals), but none had been tested for binding to the tetrad:heptad structure. The table in 6C indicates that 4 of the 12 “first generation” compounds from Cylene stabilized the secondary structure within the HER-2/neu PPT. CX11251 appeared to be the most effective compound at stabilizing the HER2/neu G-quadruplex, demonstrating DNA polymerase arrest at 0.1 uM. None of these compounds interacted with the control template oligo, indicating that polymerase arrest was sequence-specific for the secondary structure in the HER2/neu polypurine tract.

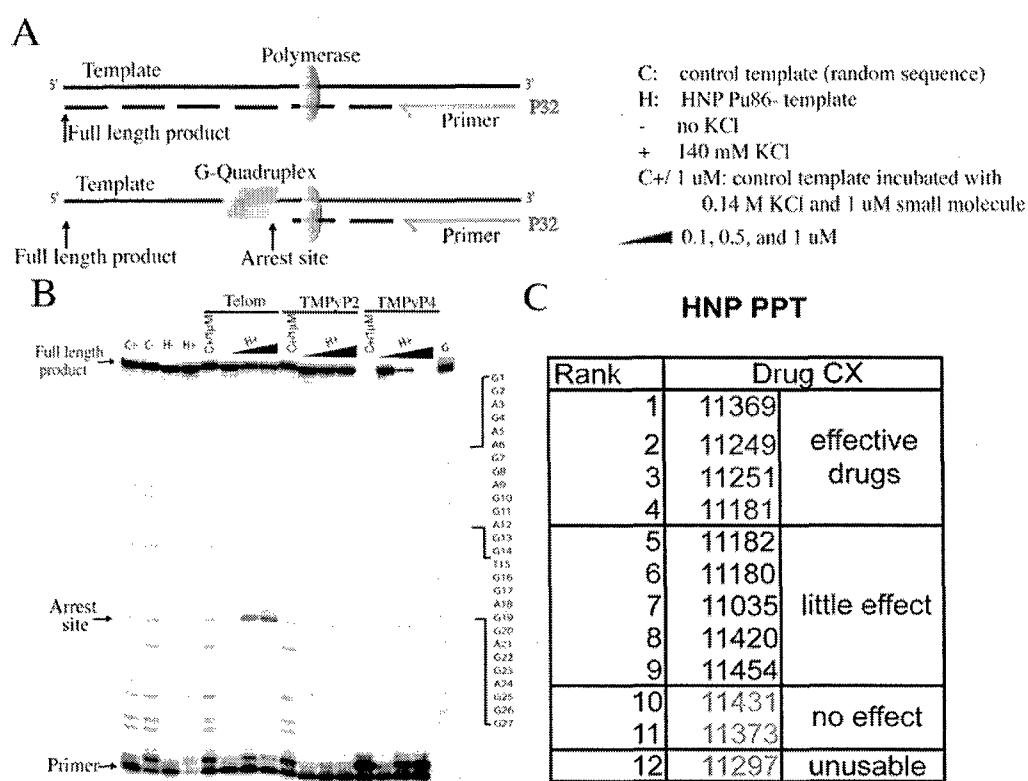


Figure 2. Evaluation of potential G-quadruplex ligand binding to the HER-2/neu promoter by the Taq Pol arrest assay.

We investigated the ability of G-quadruplex interactive compounds to suppress HER-2/neu expression in cultured cancer cells. Real-time RT-PCR was performed to evaluate the level of transcription after treating cells for 24 hours with the indicated compounds (**Figure 3**). Telomestatin modestly reduced HER-2/neu expression at non-toxic doses in the colon cancer cell line COLO-205 (top panel) that aberrantly co-overexpresses HER-2/neu and c-MYB (co-expression of c-MYB is of interest because c-MYB contains a highly homologous GGA repeat sequence in its 5' untranslated region). The porphyrins (TmPyP4 and P2) had no effect on HER-2 expression as predicted by the TaqPol assay. These observations demonstrate in principle that the Taq Pol arrest assay can be used to screen for compounds that might be useful HER-2/neu inhibitors. We also investigated the effects of a second generation of Cylene compounds on HER-2/neu expression in the breast cancer cell line MDA-MB-175-VII (bottom panel). This cell line is a good model for transcriptional overexpression of HER-2/neu, since it overexpresses HER-2/neu mRNA 10-fold from a single copy of the HER-2/neu gene. CX1398 had a significant effect on HER-2/neu transcription, decreasing expression by 70%. These data need to be confirmed in several additional cell lines and the “second generation” Cylene compounds need to be evaluated in the Taq Pol assay, but we believe that CX1398 represents a good potential lead compound for HER-2/neu transcriptional inhibition by interaction with the G-quadruplex related structure formed in the HER-2/neu promoter.

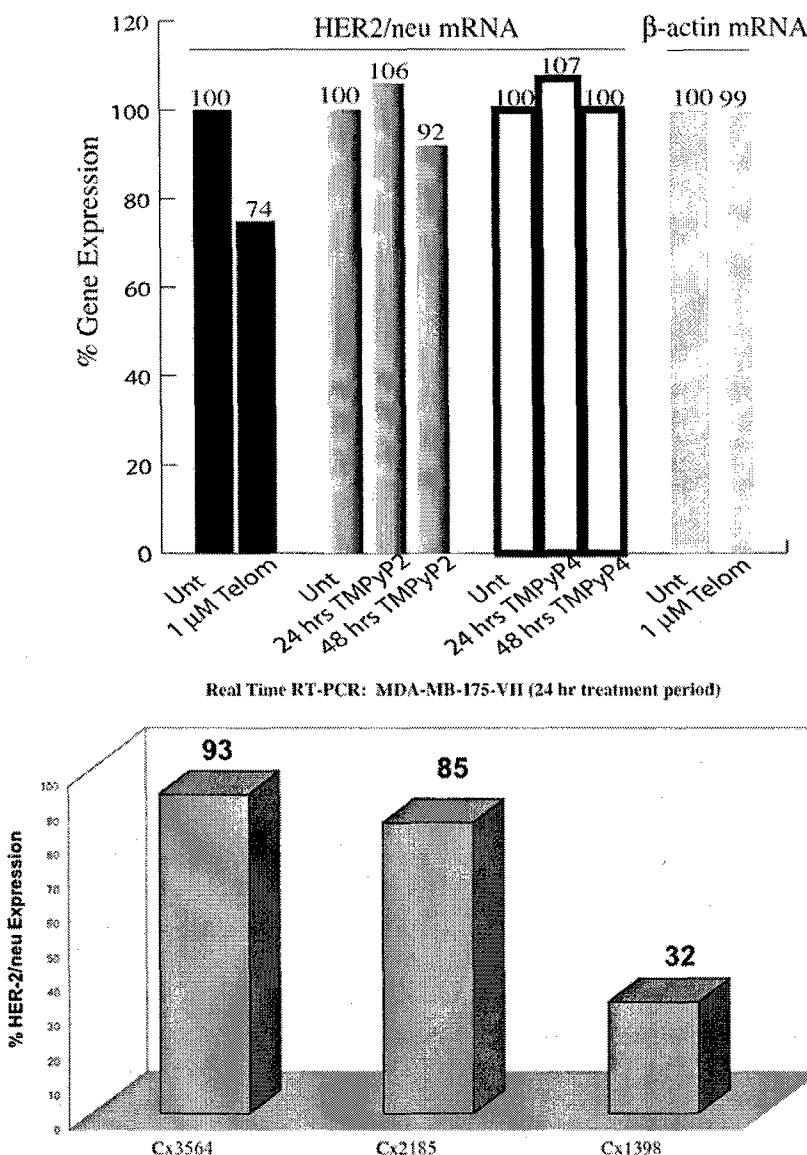
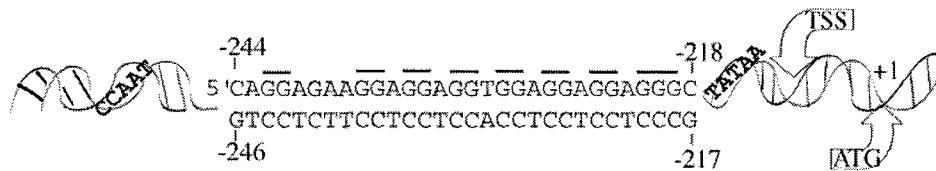


Figure 3. Real-time RT-PCR analysis of HER-2/neu expression in Colo-205 cells (top panel) and MDA-MB-175-VII cells after treatment with potential G-quadruplex interactive compounds.

We also biochemically characterized the ability of DNA oligonucleotides (“oligos”) representing the HER-2/neu PPT to form stable secondary structure(s) in solution. Oligos used in this study are illustrated in alignment with the HER-2/neu promoter PPT (Figure 4, top panel). The HER-2/neu promoter differs from the (GGA)₄ sequence characterized by NMR to form a tetrad:heptad by a single nucleotide, specifically in the final adenine, which the NMR structure showed as uninvolved in secondary structure formation. To determine if the oligo (GGA)₃GGG, representing the exact sequence of the HER-2/neu promoter, could form the same secondary structure as

(GGA)₄ we recorded circular dichroism (CD) spectra from the (GGA)₄ oligo and its variants (GGA)₃GGG and (GGA)₃GG. Figure 4 (bottom panel) compares the CD spectra of the four oligos (GGA)₄, (GGA)₃GGG, (GGA)₃GG, and a 23 bp random control sequence control. A positive CD peak at 260 nm is indicative of a parallel G-quadruplex, while a positive peak at 295 nm is indicative of an antiparallel G-quadruplex [14, 15]. The CD spectra demonstrate that at physiological concentration of potassium (140 mM), a positive CD peak around 260 nm forms for (GGA)₄, (GGA)₃GGG, and (GGA)₃GG but not for the control sequence suggesting that the 3 G-rich oligos form similar potassium dependent parallel G-quadruplex. The CD spectra are identical to the spectrum reported for the (GGA)₄ oligo by Matsugami *et al* in their initial report of the tetrad:heptad structure [2]. These observations imply that the HER2/neu promoter polypurine tract contains a sequence capable of tetrad:heptad formation.

A HER2/neu Promoter Polypurine Tract (PPT)



B Oligonucleotides

(GGA) ₈ :	5' GGAGGAGGAGGAGGAGGAGGAGGA 3'
HNPPt36:	5' TCACA GGAGAAGGGAGGAGGTGGAGGAGGG CTGC 3'
(GGA) ₄ :	5' GGAGGAGGAGGA 3'
(GGA) ₃ GGG:	5' GGAGGAGGAGGG 3'
(GGA) ₃ GG:	5' GGAGGAGGAGG 3'

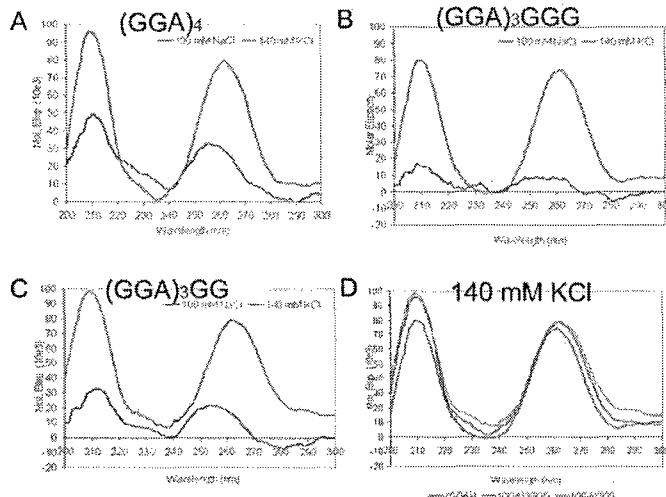


Figure 4. Top Panel. Sequences of the oligos used to biochemically characterize the structure of the HER-2/neu promoter. **Bottom Panel.** CD spectra of (GGA)₄ and variant oligos in the presence and absence of KCl, A -C. The spectra of the oligos in KCl are superimposed in D.

Nondenaturing gel electrophoresis was performed to investigate the electrophoretic mobility shift (EMSA) of an oligo representing the HER-2/neu PPT (**Figure 5**). The (GGA)₄ oligo and variants did not form altered mobility complexes, indicating that EMSA is not informative for these very short oligos. We compared the mobility of the (GGA)₈ oligo, which forms a tetrad:heptad:heptad:tetrad structure stabilized by stacking interactions of two heptads [16] to the mobility of the entire PPT of the HER-2/neu promoter plus immediate flanking sequence (Pu36). In potassium (lanes 2, 4, and 6), the (GGA)₈ oligo forms predominantly a rapidly migrating species consistent with an intramolecular G-quadruplex, whose compact structure allows it to migrate rapidly in the gel, while in the absence of potassium (lanes 1, 3, and 5), the predominant species migrates as random coil DNA (**Figure 5A**). The electrophoretic mobility of Pu36 is complex (**Figure 5B**). Slow mobility structures are predominant, and indicate the probable formation of intermolecular complexes, which could be tetrad:heptad dimers from the stacking interaction between two heptads (see **Figure 1**), but more likely represent parallel and anti-parallel tetrameric G-quadruplexes formed from the association of 4 strands of Pu36 in solution. In potassium (lanes 2, 4, and 6), Pu36 also forms a high mobility species which likely represents an intramolecular G-quadruplex or tetrad:heptad. This high mobility species is favored at low concentrations of Pu36, which would tend to lessen the formation of intermolecular complexes. DMS treatment of the oligo to methylate the N7 of guanines prevented the formation of the altered mobility complexes. Since the N7 of guanine is involved in Hoogsteen hydrogen bonding, these experiments provide further evidence that these structures are G-quadruplexes.

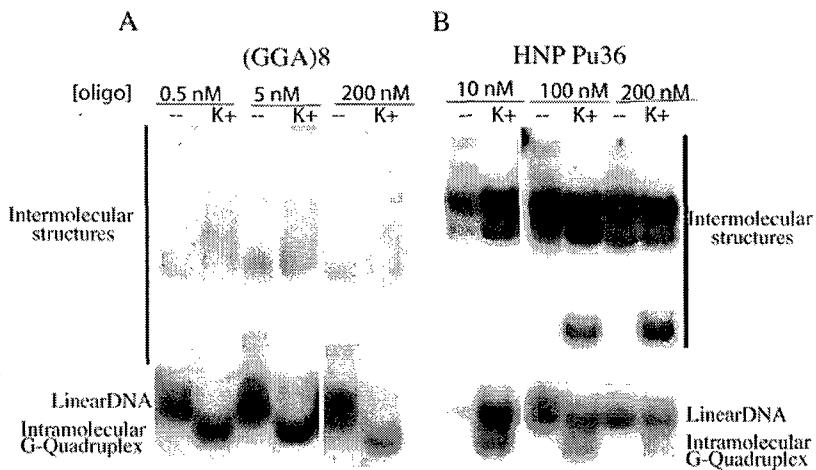
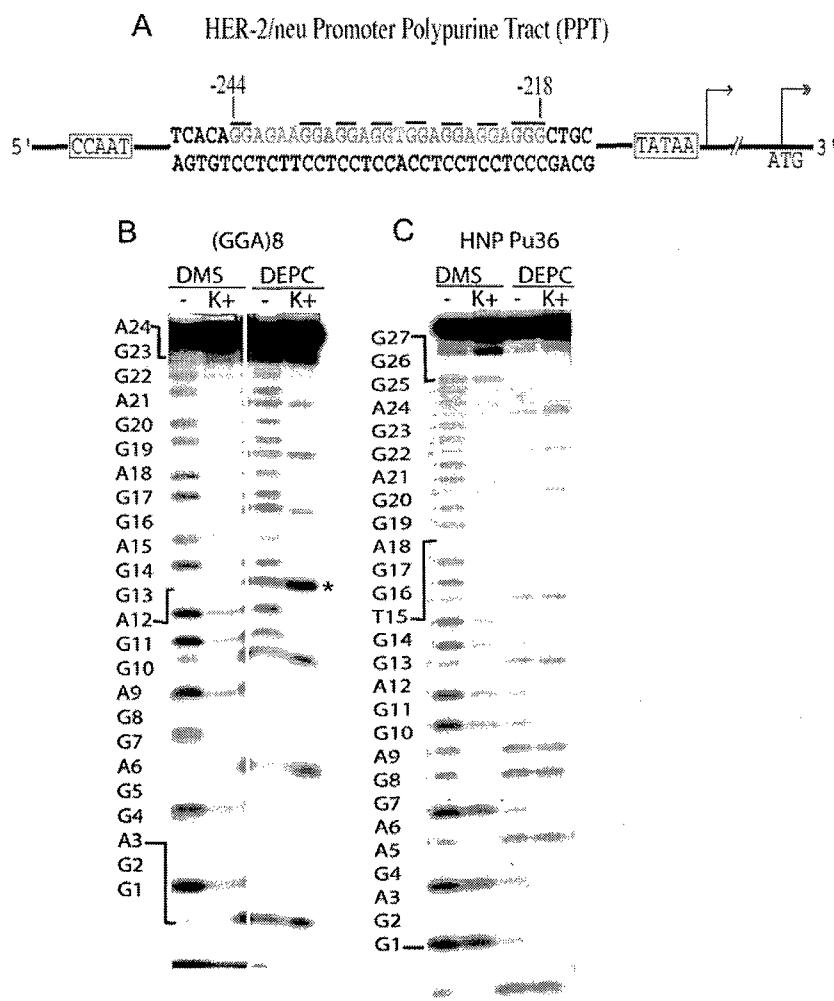


Figure 5. Electrophoretic mobility of the proven tetrad:heptad forming (GGA)₈ oligo compared to the PPT of the HER-2/neu promoter at various concentrations of the oligos and in the presence or absence of 140 mM KCl.

Chemical footprinting was performed to further characterize the G-quadruplex formed by the HER-2/neu PPT (**Figure 6**). The (GGA)₈ control oligo was used to obtain DMS (G-probe) and DEPC (A-probe) protection patterns indicative of a tetrad:heptad (**Figure 6B**), and demonstrates protection of all guanines and adenines predicted by the

NMR structure to be used for tetrad:heptad formation. The DEPC footprint is also in agreement with the NMR structure because the A12 residue linking the two heptads is more reactive with DEPC compared to other adenines (see **Figure 1**). Pu36 produces a potassium dependent DMS footprinting pattern characterized by partial protection of Gs at the distal (3') end of the PPT and hypersensitivity of adenines (As) between G doublets in the distal GGA repeats (**Figure 6C**). The footprint involves G16-G26 and G13-G14 with partial protection of G7-8 and G10-11. G16-G26 are the guanines that we predict to be involved in tetrad:heptad, and the extended footprint may indicate that competing structures are formed (as shown in the EMSAs) or that some of the guanines flanking the tetrad:heptad forming unit of the HER-2/neu PPT can contribute to stabilizing the secondary structure formed by this sequence. Adenines in the Pu36 oligo exhibit unequal reactivity with DEPC in a pattern which is difficult to reconcile with tetrad:heptad formation by this oligo. In summary, short oligos representing the distal end of the HER-2/neu PPT have CD spectra that are identical to the spectrum of a tetrad:heptad, but longer oligos have complex electrophoretic mobility and DMS and DEPC protection patterns that are probably derived from competing intermolecular structures in solution.

**Figure 6. DMS and DEPC footprinting of the HER-2/neu PPT.**

Key Research Accomplishments (bulleted list)

- Screening compounds with Taq Pol arrest assay identified several potential HER-2/neu transcriptional inhibitors.
- Demonstration that compounds identified by this assay can suppress HER-2/neu expression in cells.
- Identification of a lead compound, CX1398 for further evaluation against various breast cancer cell lines.
- Biochemical characterization of the HER-2/neu promoter in solution
 - Circular dichroism studies demonstrate identity with the NMR tetrad:heptad structure
 - EMSAs and footprinting studies demonstrate competing structures in solution

Reportable Outcomes (reprints, presentations, patents, etc.)

Y. Krotova, M. Boros, R. Memmott, A. Ziembka, L. Hurley, and S. Ebbinghaus, Transcriptional Control of the HER-2/neu Promoter By DNA Secondary Structure, Era of Hope Department of Defense Breast Cancer Research Program Meeting, 2005

Conclusions

We conclude that the GGA repeats in the HER-2/neu promoter can form a stable secondary structure known as a tetrad:heptad, although the biochemical characterization of the secondary structure formed by the full length of the HER-2/neu PPT has proven difficult because of competing secondary structures by these G-rich oligos. Nonetheless, we have demonstrated in principle that we can use a simple DNA polymerase arrest assay to screen compounds that might be useful inhibitors of HER-2/neu transcription, and we have identified at least one potential novel lead compound to investigate further in the next two years of the grant. The ambiguities of the biochemical studies presented in this report will require further work to resolve, as we initially predicted and proposed. For our future biochemical studies, we will make use of oligos with G-quadruplex disrupting "mutations" (substitutions of guanines with the 8-aza-7-deaza or ppG analog of guanine). We will also begin evaluating the effect of G-quadruplex interactive compounds on HER-2/neu promoter luciferase constructs with and without G-quadruplex disrupting mutations to determine whether the inhibition of HER-2/neu expression by CX1398 and other compounds that we will test is due to interaction with the quadruplex forming region of the HER-2/neu promoter. These future studies continue to follow the outline of proposed studies that we initially presented in our statement of work.

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Appendix

none